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ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 67 (2007) 320–328
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Re-dispersible cationic solid lipid nanoparticles (SLNs) freeze-dried without cryoprotectors: Characterization and ability to bind the pEGFP-plasmid

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Received 25 September 2006; accepted in revised form 1 February 2007
Available online 14 February 2007

Abstract

Cationic solid lipid nanoparticles (SLNs) have recently been suggested for non-viral gene delivery as a promising alternative to the liposomes. The aim of this study was to investigate the possibility to obtain re-dispersible cationic SLNs after a freeze-drying process in the absence of lyo- and/or cryoprotectors. The physical-chemical characteristics of cationic SLNs and their ability to bind gene material were investigated before and after the freeze-drying. To perform this study three samples of cationic SLNs, based on stearic acid, Compritol or cetylpalmitate, were prepared and characterized by PCS (photon correlation spectroscopy) and AFM (atomic force microscopy). The results indicated that solely the re-dispersed sample of stearic acid (SLN-SA) became very similar in terms of size and morphology to the fresh prepared sample, although it displayed a sensible reduction of the zeta potential (from 39.2 to 23.3 mV). By both the DSC (differential scanning calorimetry) and the ESCA (electron spectroscopy for chemical analysis) determinations, the reduction of the zeta potential was ascribed to the loss of the cationic lipids from the particle surface due to the rearrangement of the stearic acid lattice after the freeze-drying. Finally, the gel electrophoresis analysis demonstrated that SLN-SA re-suspended in PBS are unable to complex the DNA, while the SLN-SA re-dispersed in water displayed the same ability to bind DNA as the fresh prepared sample. We can conclude that cationic SLNs, based on stearic acid, retain the ability to complex DNA even after the freeze-drying in the absence of lyo- or cryoprotectors; thus, the powder form of this sample represents an attractive candidate to be investigated as in vivo DNA vector formulation.

Keywords: Solid lipid nanoparticles; Freeze-drying; Gel electrophoresis; Differential scanning calorimetry; Atomic force microscopy; pEGFP-plasmid

1. Introduction

In the recent years, increasing attention has been paid to solid lipid nanoparticles (SLNs) as alternative colloidal carrier systems to polymeric nanoparticles, liposomes and nanoemulsions. SLNs are composed of a high melting point lipid as a solid core, coated by surfactants [1,2]. Referring to SLNs, the term lipid is used in a broad sense and applies for triglycerides (i.e. tristearin), partial glycerides (i.e. Compritol), fatty acids (i.e. stearic acid), steroids (i.e. cholesterol) and waxes (i.e. cetylpalmitate) [2].

The increasing interest gained by the SLNs as a colloidal drug carrier is due to their properties: reduced effect of ionic strength on stability, possible targeting by suitable chemical modification, good protection of encapsulated bio-active molecules, high encapsulation loads, absence of carrier bio-toxicity, avoidance of toxic organic solvents, ease of scale-up procedures and low cost [1,2]. Therefore, SLNs have been proposed as drug delivery systems of several drugs such as clozapine [3], diazepam [4], cyclosporine [5], ibuprofen [6], etc. Moreover, cationic SLNs have been

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doi:10.1016/j.ejpb.2007.02.006

recently proposed in the gene therapy as non-viral transfection agents [7]. The common ground of cationic liposomes [8,9], nanoemulsions [10,11] and SLNs for transfection is the need for cationic lipids to facilitate the deoxyribonucleic acid (DNA) binding. Recently, SLNs and liposomes, formulated from the same cationic lipids, have shown equipotent in vitro transfection efficiencies [12]. With respect to liposomes, the main advantage of the SLNs is their physical and chemical long-term stability. Indeed, optimized SLNs are found physically stable for 12–24 months [13,14]. However, this stability is not a general feature of SLN dispersion, because in most cases, an increase in particle size can be observed in a shorter period of time [15]. To overcome these problems, the freeze-drying process represents a well-established approach to increase chemical and physical SLN stability over extended time periods [16]. The investigation of the freeze-drying modalities of SLNs (especially negatively charged or neutral nanoparticles) has been performed thoroughly [16–19], while only few examples of successful freeze-dried cationic nanoparticles are reported in the literature [20,21]. In these studies the role of lyoprotective and/or cryoprotective agents (such as polyvinylpyrrolidone, sorbitol, mannose, threalose, glucose) in preventing the particle aggregation and improving the re-dispersion of the dry product has been well-established [16,17,19]. Moreover, important interactions between lyoprotective agents and cationic nanoparticles, influencing nanoparticle biological activity (i.e. cell transfection), have been reported [20].

Therefore, the aim of this study was to evaluate the possibility to obtain re-dispersible cationic SLNs after freeze-drying without any lyoprotective and/or cryoprotective agents. Moreover, the influence of the freeze-dry process on physicochemical characteristics of SLNs as well as on their ability to bind gene materials is investigated. To perform this study three samples of cationic SLNs were prepared and characterized before and after the freeze-dry process. Finally, the DNA-binding capacity was also compared before and after the freeze-drying process, as a measure of the biological activity.

2. Materials and methods

2.1. Materials

Cetylpalmitate was purchased from Acros Organics (Geel, Belgium). Pluronic F68 and sodium cholate were provided by Sigma–Aldrich Chemie (Deisenhofen, Germany). Compritol 888 ATO (glycerol behenate) was a gift from Gattafossè (Weil am Rhein, Germany). Stearic acid was purchased from Carlo Erba Reagenti (Milan, Italy). Esterquat 1 (EQ1) (*N*,*N*-di-(β-stearoylethyl)-*N*,*N*-dimethyl-ammonium chloride) was a gift from Gerbu Biotechnik (Gaiberg, Germany). Stearylamine (ST) was provided by Fluka (Deisenhofen, Germany). Agarose GellyPhor, Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffer (PBS) and other culture reagents were purchased from Euroclone Celbio (Milan, Italy). All other chemical reagents were obtained commercially as reagent-grade products.

2.2. SLN formulation

The cationic SLNs were produced by a modification of the oil-in-water microemulsion methods [22]. Briefly, the solid component (250 mg of cetylpalmitate, stearic acid or compritol) was melted at around 10 °C above its melting point. At the same time, an aqueous solution (10 mL) containing stearylamine (3% w/v), EQ1 (1% w/v) and Pluronic F68 (2% w/v) was heated at the same temperature of the melted mass. Then, the melted mass of the solid component was dispersed in the aqueous solution using a high-speed stirrer (Ultra-Turrax T25, IkaWerk, Staufen, Germany) at 20,500 rpm for 5 min to form a hot O/W emulsion. Then, the hot emulsion was cooled at 4 °C by an ice bath, maintaining the mechanical stirring at 20,500 rpm for 10 min, i.e. until the formation of the solid lipid nanoparticle (SLN) suspension. This suspension was purified and concentrated by vacuum ultra-filtration using a polycarbonate holder (Sartorius, Goettingen, Germany) equipped with a Polyethersulfone (PESU) filter (cut-off of 100 nm, Sartorius). The final concentration of the SLN in the purified suspension was 20 mg/mL ± 4%. This value was determined from the yield of the freeze-drying process. The purified SLN suspension (10 mL) was separated into two fractions: the first one (fresh prepared SLNs) was stored at 4 °C in dark glass and the second one (freeze-dried SLNs) was freeze-dried as described below.

SLNs without the cationic lipids were prepared using the same technique described above, but adding in the water phase (10 mL) 370 mg of sodium cholate in the place of the cationic lipids (stearylamine and EQ1).

All the batches were prepared at least in triplicate.

2.3. Freeze-drying/re-dispersion of cationic SLNs

The second fraction of the SLN samples was frozen at −20 °C overnight in a glass vial. Then, the sample was moved to the freeze-drier (Heto-Holten A/S, Allerød, Denmark) and the freeze-drying process was carried out at −55 °C at a pressure of 10^{-4} Torr during 36 h. Then, the SLN powder was collected and stored at 4 °C. The re-dispersion of the freeze-dried samples (10 mg) was performed in deionised water (2 mL) by three cycles of vortex (30 s) (Zx^3, Velp Scientifica, Milan, Italy) followed by a treatment in an ultrasound bath (Sonorex™, Bandelin, Mörfelden, Wan, Germany) (30 s).

2.4. PCS (photon correlation spectroscopy) experiments

The PCS experiments were carried out on both the fresh prepared SLN samples and the re-dispersed freeze-dried samples. A Zetasizer Nano ZS (Malvern, Worcs, UK) equipped with a 4 mW He–Ne laser (633 nm) was used for the size determination. Each experiment was carried

out at 25 °C using deionised water with a refraction index of 1.33, a viscosity of 0.8872 cP and repeated three times for each samples. The data are expressed as mean values ± standard deviation.

Similarly, the zeta potential was measured using the same equipment described previously with a combination of laser Doppler velocimetry and a phase analysis light scattering (PALS). Each experiment was repeated three times. Data are expressed as mean values ± standard deviation.

2.5. Atomic force microscopy (AFM)

AFM observation was performed by a Park Autoprobe Atomic Force Microscope (Park Instruments, Sunnyvale, CA, USA). AFM images were obtained by measurement of the interaction forces between the tip and the sample surface [22]. The experiments were conducted in water at room temperature (20 °C) and at atmospheric pressure (760 mmHg) operating in non-contact mode (NC-AFM). Triangular silicon tips were used for this analysis. The resonant frequencies of this cantilever were found to be about 120 KHz. Immediately before the analysis, the samples (fresh prepared or re-dispersed SLNs) were diluted in water (1:100 v/v) to obtain a less sticky fluid and deposited (40 μl) onto a small mica disk with a diameter of 1 cm. After 2 min, the excess of water was removed using paper filter. The mean size of SLNs by AFM was obtained processing the topographical AFM images with the ProScan Data Acquisition software.

2.6. DSC analysis

Differential Scanning Calorimetry (DSC) analysis was performed using Netzsch DSC 200PC (Netzsche, Selb, Germany). The instrument was calibrated with indium (calibration standard, >99.999%) for melting point and heat of fusion. A heating rate of 2 °C/min was employed in the range of 25–90 °C. Analysis was performed under nitrogen purge (20 mL/min). The samples were weighted into standard aluminum pans and an empty pan was used as reference. The freeze-dried sample was analysed the day after the production. Before the analysis, the fresh prepared SLN-SA was dehydrated under vacuum (10 mmHg) for 48 h at 25 °C.

2.7. Electron spectroscopy for chemical analysis (ESCA) studies

In order to determine the difference in the distribution of the atoms on the surface of the particles, the electron spectroscopy for chemical analysis (ESCA) was performed on the selected SLN-SA sample (see Table 1) either as fresh prepared or as re-dispersed freeze-dried powder. Moreover, a spectrum was also recorded from a fresh prepared sample formulated as the SLN-SA batch but without adding cationic components (SLN-ncSA sample). The spectra were recorded on an analysis system 04-153 X-ray source (PHI, Uvalca-PHI, Tokyo, Japan) and an hemispherical electron analyser EA11 (Leybold Optics, Germany) by MgK 1,2 radiations ($E = 1253.6$ eV). The analysis was carried out in FAT (fixed retardation ratio) mode with 190 eV pass energy. The pressure in the sample analysis chamber was ca. 10^{-9} mbar. Data acquisition and processing were performed using the RBD AugerScan 2 program.

2.8. Plasmid DNA production

The plasmid vector expressing Enhanced Green Fluorescent Protein pEGFP-C3 was purchased from Invitrogen (Carlsbad, California, USA) and transformed into *Escherichia coli* (XL_1 Blue R). A Maxiprep from 500 mL of overnight culture was performed with Qiagen Kit according to the manufacturer's instruction (Endofree Maxi Prep, QIAGEN, Hilden, Germany).

2.9. Agarose gel electrophoresis

SLN-SA as fresh prepared suspension or as freeze-dried powder was complexed with the plasmid DNA and analysed by agarose gel electrophoresis. The re-dispersion of the freeze-dried powder was accomplished, according to the method described in Section 2.3, using either deionised water or PBS (phosphate buffer solution 20 mM, pH 7.4).

Both the fresh prepared suspension and the re-dispersed powder (in water or in PBS) were added to plasmid DNA in the ratios indicated in the various experiments and incubated for 45 min at room temperature.

After the incubation, the SLN:DNA mixtures were diluted in water or in serum free medium (DMEM).

The analysis by agarose gel electrophoresis was performed at 50 V for 4 h in 0.8% (w/v) agarose gel in TBE buffer (0.045 M Tris–borate, pH 8.3 and 0.001 M EDTA) after adding to each sample the loading buffer (40% v/v glycerol and 0.25% w/v bromophenol blue in TBE buffer). The results of DNA migration were visualized under UV light, after staining with ethidium bromide. The total amount of DNA in each lane was 0.5 μg for all the samples.

3. Results and discussion

3.1. Size and zeta potential of the fresh prepared SLN suspension

SLNs were successfully produced by a modification of the oil-in-water microemulsion methods [22]. After the preparation, all the samples were purified by ultra-filtration in order to remove the excess of the surfactant (Pluronic F68). Table 1 summarizes components, size and zeta potential of the cationic and not cationic samples. The results indicate that the three cationic samples displayed an average size between 160 and 209 nm, according to their composition. However, a polydispersion of the particles was obtained, being the PDI (polydispersion index) value in the range of 0.2–0.4 for all the SLN lipid matrixes

Table 1
Components, particle size and zeta potential of fresh prepared SLNs obtained in absence (not cationic SLN) and in presence of the cationic lipids

Formulation	Components	Particle size ± S.D. (PDI)	Zeta potential (mV) ± S.D.
SLN-ncCp	Cetylpalmitate sodium cholate	270 ± 70 (0.601)	−18.3 ± 6.0
SLN-ncSA	Stearic acid sodium cholate	470 ± 90 (0.385)	−22.2 ± 6.5
SLN-ncCom	Comprito sodium cholate	420 ± 100 (0.402)	−32.2 ± 10.0
SLN-Cp	Cetylpalmitate EQ1 stearylamine	193 ± 54 (0.396)	+ 42.7 ± 10.1
SLN-SA	Stearic acid EQ1 stearylamine	209 ± 23 (0.219)	+ 39.9 ± 9.7
SLN-Com	Compritol EQ1 stearylamine	160 ± 13 (0.389)	+ 34.8 ± 8.7

investigated. The presence of a slight percentage (<0.5%) of microparticles with a size of approximately 4 μm is probably responsible for the observed polydispersion of the samples. The samples prepared in absence of cationic components (SLN-nc) demonstrated a bigger mean diameter and a higher PDI value (Table 1). These results indicate that both the cationic lipids could also exert a surfactant activity during the preparation of the particles.

The stability of the SLN suspensions was determined by the evaluation of the size and the zeta potential of the particle suspension stored for two months in the dark at 4 °C. Fig. 1 shows the results obtained from the fresh SLNs without cationic lipids as soon as prepared and 7, 15, 30 and 60 days after the preparation. For all the three samples both zeta potential and dimensions remain stable during the time period of the analysis. Fig. 2 shows the results obtained from the fresh prepared cationic SLNs during the same range of time period. The SLN-Cp (constituted of cetylpalmitate) appeared practically stable in size during 15 days. However, after 7 days a marked reduction of the zeta potential value was observed. The SLN-SA (constituted of stearic acid) displayed a good stability of the size and of the zeta potential during 30 days, then, a progressive reduction of the zeta potential with an increase of the size was observed. Finally, the SLN-Com (constituted of Compritol) became the less stable sample because, after only 7 days, a gradual increase of the mean diameter of the particles occurred, indicating the start of the formation of aggregates. Simultaneously, a fast decrease of the zeta potential value was observed.

Therefore, all the cationic SLNs displayed an unsatisfactory stability when stored as suspension. It is known the SLN tendency of agglomeration during storage as liquid formulation was less marked for particles formulated in absence of cationic components [22]. Indeed, the presence of the cationic components in the lipid matrix exerts a negative effect on the suspension stability, probably owing to the modification of the surface properties of the particles and the shifting of the particle charge during the storage.

The inherent instability observed for the cationic SLN suspension is a severe limitation for the practical application of these carriers in medicine. Therefore, in order to obtain more stable vectors, the cationic SLNs were freeze-dried in absence of both lyo- and cryoprotective agents.

3.2. Size, zeta potential and AFM analysis of the re-dispersed cationic SLNs

The freeze-dried cationic SLNs were re-dispersed in deionised water as described in Section 2.3; the size and the zeta potential of the reconstituted SLNs are indicated in Table 2. Comparing these data with the data reported in Table 1, no modification in the size for the SLN-Cp and SLN-SA samples is evident. On the contrary, the

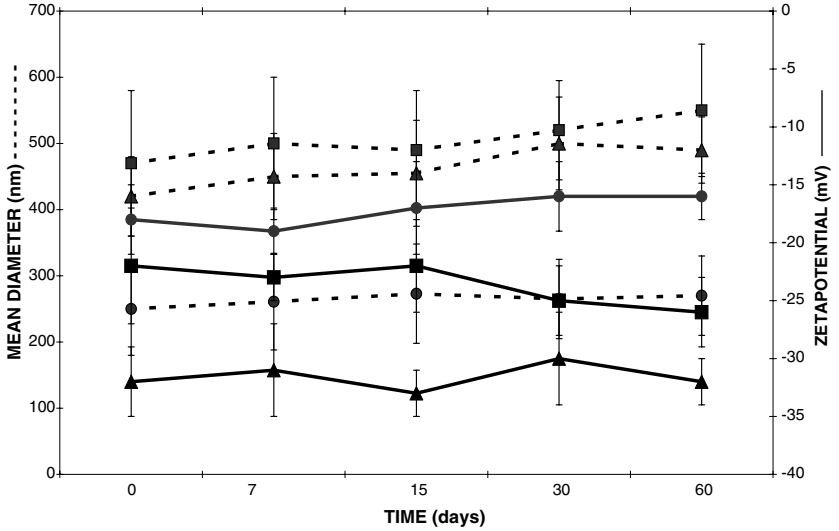

Fig. 1. Time stability evaluation of size and zeta potential of the fresh prepared SLN without cationic lipids. Size: (---●---SLN-ncCp; ---■---SLN-ncSA; ---▲---SLN-ncCom); zeta potential: (—●—SLN-ncCp; —■—SLN-ncSA; —▲—SLN-ncCom).

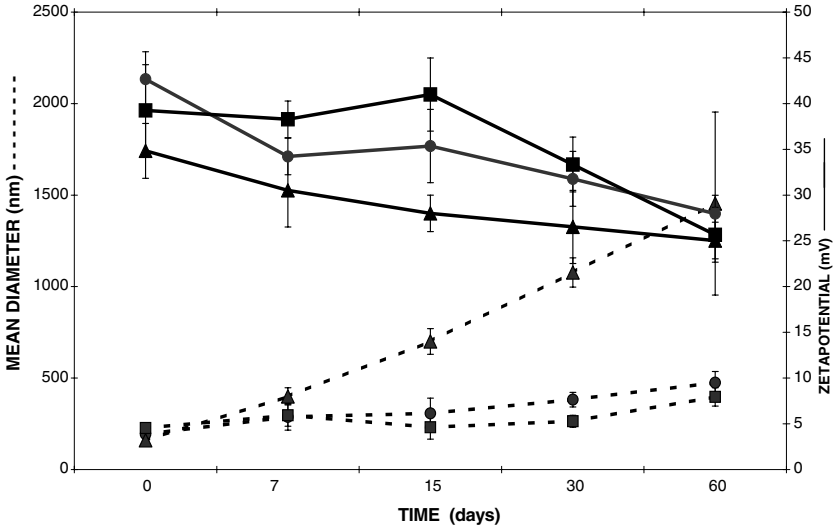

Fig. 2. Time stability evaluation of size and zeta potential of the fresh prepared cationic SLN. Size: (---●---SLN-Cp;---■---SLN-SA; ---▲---SLN-Com); zeta potential: (—●—SLN-Cp; —■—SLN-SA; —▲—SLN-Com).

Table 2
Size and zeta potential of freeze-dried cationic SLNs, after reconstitution in deionised water

Sample	Particle size (nm) ± S.D. (PDI)	Zeta potential (mV)
SLN-Cp	205 ± 54 (0.288)	+ 18.9 ± 0.6
SLN-SA	233 ± 62 (0.28)	+ 23.3 ± 3.1
SLN-Com	304 ± 70 (0.246)	+ 25.8 ± 1.3

SLN-Com showed a noticeable increase in the mean particle size (from 160 to 304 nm). As concerns the zeta potential value, all the samples showed a decrease of the surface charge; in particular the SLN-Cp showed the most pronounced decrease of the zeta potential (from 42.7 to 18.9 mV).

Therefore among the three samples, the SLN-SA became the most stable sample in terms of size and zeta

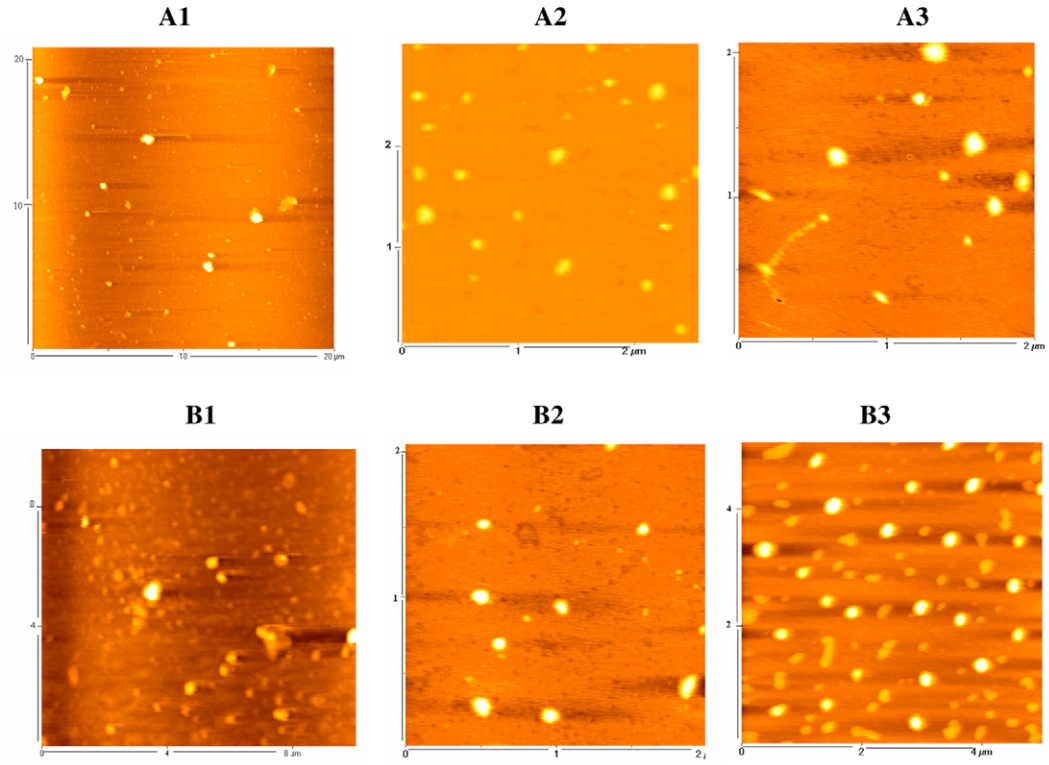

Fig. 3. Atomic force microscopy of SLN before (A1–A3) and after (B1–B3) the freeze-dry process. Fresh prepared sample: (A1: SLN-Cp; A2: SLN-SA; A3: SLN-Com). Freeze-dried SLN, after reconstitution (B1: SLN-Cp; B2: SLN-SA; B3: SLN-Com).

potential after the freeze-dry process, indicating the stearic acid as the lipid material with the better reconstitution properties. In order to confirm these data, the AFM analysis of the three SLN samples was performed before and after the freeze-drying process (Fig. 3).

All the fresh prepared samples (Fig. 3,A1–A3) presented a regular shape with a broad size range. Moreover, the observed nanoparticles appeared only 60-80 nm in height: thus, the particles can be described as platelet shaped. However, processing the AFM images, the diameter of particles became larger (by about 20%, data not shown) with respect to the values obtained by the PCS technique. Therefore, the noticeable difference between width and height can be in part attributable to the cantilever probe of the AFM that pushing and warming the particles may determinate a deformation of their original morphology [23].

As concerns AFM analysis of re-dispersed freeze-drying samples (Fig. 3,B1–B3), the SLN-Cp and SLN-Com displayed particles with an irregular morphology and/or a bigger diameter respect to the fresh prepared samples, probably due to the formation of aggregates. On the contrary the particle of the SLN-SA after freeze-drying appeared with the same morphology as before.

As a consequence, among the three considered samples (Table 1), solely the re-dispersed SLN-SA became very similar in terms of size and morphology to the fresh prepared sample. Based on these results, the SLN-SA was selected as promising freeze-dried sample for further physicochemical characterization aimed to explain the reduction of the surface charge following the freeze-dry process.

3.3. DSC analysis

During the freeze-drying process in the absence of lyo- and cryoprotectors, modification of the physical state of the SLN components should occur. Differential scanning calorimetry (DSC) is extensively used to investigate the physical status of the lipids, because several lipid modifications possess different melting points and melting enthalpies [2].

In Fig. 4A are reported the thermograms obtained from each single component of the SLN-SA; Fig. 4B reports the curves obtained from the blend of the lipid components and from the SLN-SA before and after the freeze-drying. Table 3 shows the related data of peaks shown in Fig. 4.

The DSC curve of the lipid blend (Fig. 4B) revealed two peaks: the first one at 53.2 °C with an energy of 207.6 J/g, attributable to the mixture of stearic acid and stearylamine and the second one at 68.0 °C with an energy of 20.6 J/g, attributable to the EQ1.

The fresh prepared SLN-SA displayed a single peak at 49.4 °C with a very low enthalpy (32.2 J/g), while the freeze-dried SLN-SA showed a peak at 44.9 °C with an enthalpy of 66.2 J/g. Moreover, in both cases, no transition temperature, attributable to the EQ1 melting point, was registered.

The noticeable depression of the melting point of the SLN-SA samples (both before and after the freeze-drying)

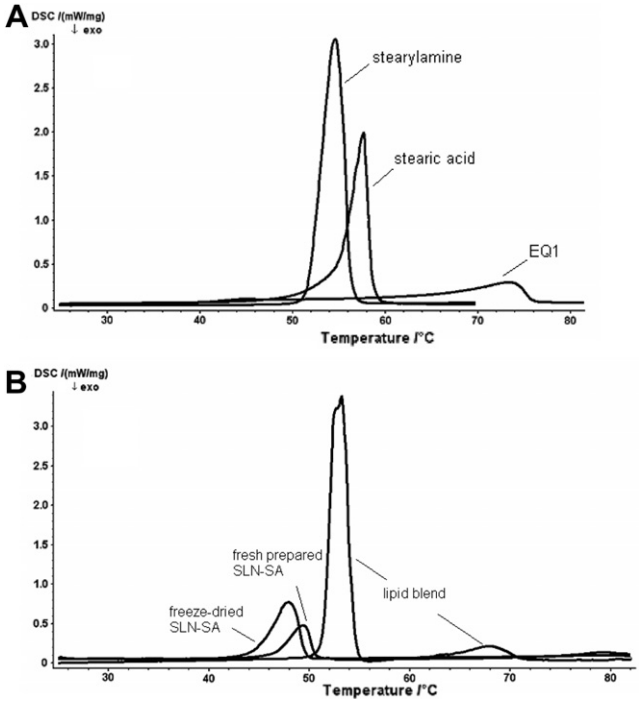

Fig. 4. DSC thermograms of: bulk stearylamine, stearic acid and EQ1 (A); lipid blend, fresh prepared SLN-SA and freeze-dried SLN-SA (B).

Table 3
Onset temperature, T max, and enthalpy of each DSC peak

Samples	Onset temperature	T max (°C)	Enthalpy (J/g)
Stearic acid	54.8	57.7	163.7
Stearylamine	51.8	54.6	258.4
EQ1	67.6	73.3	92.1
Blend of SLN-SA components	51.5	53.2	207.6
	67.7	68.0	20.6
Fresh prepared SLN-SA	46.6	49.4	32.2
Freeze-dried SLN-SA	44.7	47.9	66.2

with respect to the lipid blend might be due to the small particle size (nanometer range) and to the high specific surface area. This effect (Kelvin effect) is described in the literature [3,24–26]. The decrease of enthalpy of the melting transition can be related to the crystallinity of SLN-SA matrix. Indeed, the crystallinity of SLNs can be calculated by the ratio of SLN-SA enthalpy to bulk stearic acid enthalpy [15,26]. Enthalpy of SLN-SA was calculated taking the enthalpy of bulk stearic acid at 163.7 J/g as 100%. Crystallinity of SLNs was surprising in this order: freeze-dried SLN-SA (40.6%) > fresh prepared SLN-SA (19.7%). This means that following the freeze-drying, in contrast with the expectative, the SLNs presented a higher crystallinity degree. It is important to notice that the numerous studies about polymorphic transitions or physical state of lipids in SLNs have been performed using freeze-dried SLNs. Therefore, very poor information are available about the state of the lipid in SLN before the freeze-drying process. From the results reported above,

the SLN-SA preparation by the oil-in-water microemulsion method (consisting in the melting of the stearic acid followed by its dropping in an aqueous solution) leads to a disordered configuration of the stearic acid. The highly disordered configuration of lipids can be correlated to their high capacity to incorporate guest molecules [2,26]. As a consequence, the stearic acid configuration in the fresh prepared SLN-SA could allow more space to accommodate stearylamine and EQ1 in the lipid matrix. Following the freeze-drying, an increase of the crystallinity degree of lipid in SLNs occurred. This rearrangement of the crystal lattice in favor of thermodynamically stable configuration might be connected to the expulsion of incorporated molecules [2,16]. In this particular case, as the incorporated molecules are represented by the cationic lipids (stearylamine and EQ1) the rearrangement of the crystal lattice can be responsible for the loss of the cationic lipids. This consideration can explain why the re-dispersed freeze-dried SLN displayed a less positive charge with respect to the fresh prepared suspension.

Therefore, in order to confirm this hypothesis, an X-ray spectroscopy analysis of the SLN-SA surface was performed.

3.4. Electron spectroscopy for chemical analysis (ESCA)

The electron spectroscopy for chemical analysis (or X-ray spectroscopy analysis) (ESCA) is a very useful technique to determine the elementary composition of particle surface; thus this technique was employed to visualize any modifications of the cationic lipid molecules on the particle surface.

Fig. 5 displayed the survey spectra obtained from a SLN sample based on stearic acid and prepared in absence of cationic components (i.e. stearylamine and EQ1). This sample (SLN-ncSA) was analysed as fresh prepared suspension after dehydration in desiccator for 48 h at 25 °C. In this case, the atomic composition determined by the ESCA (Table 4) gave a percentage of C and O (89.7 and 10.3, respectively) that was in quantitative agreement with the theoretical composition. Obviously, ESCA did not show the presence of N peak for this sample.

The ESCA-analysed atomic composition of the cationic SLN-SA was performed on the fresh prepared suspension and on the freeze-dried powder (Fig. 6A and B, respectively). These survey spectra showed, as expected, the presence of carbon, oxygen and nitrogen in the relative percentage of 88.5%, 5.2% and 2.4%. It is relevant to notice that the percentage of N corresponded to the theoretical calculation indicating the almost complete dislocation of cationic components on the surface of the fresh prepared SLNs.

Regarding the freeze-dried powder, a decrease of the N percentage was observed indicating a lower presence of the cationic lipids on the surface of the re-dispersed freeze-dried sample. This finding fits well with the zeta potential value that reveals the low positive charge of this sample (Table 2); moreover this result is consistent with the data obtained from the DSC analysis, suggesting a rearrangement of the crystal lattice of the stearic acid following the freeze-drying and the possible loss of the cationic lipid integrated in the SLN matrix.

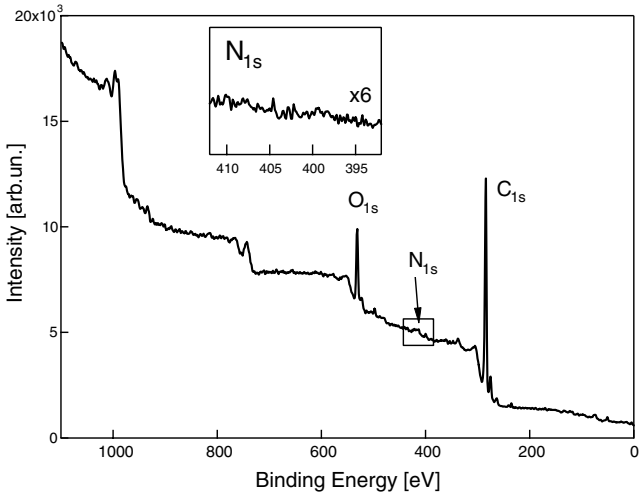

Fig. 5. ESCA spectrum of fresh prepared not-cationic SLN constituted of stearic acid (SLN-ncSA).

Table 4
Theoretical and actual (from quantitative analysis of the ESCA spectra) percentage of carbon, oxygen and nitrogen on the surface of non-cationic sample (SLN-ncSA) and cationic sample (fresh prepared or reconstituted SLN-SA)

Formulation	Theoretical[a] %			Actual[b] %		
	%C	%N	%O	%C	%N	%O
Fresh prepared SLN-ncSA	90	0	10	89.7	0	10.3
Fresh prepared SLN-SA	91.6	2.6	5.8	88.5	2.4	5.2
Reconstituted SLN-SA	91.6	2.6	5.8	91.8	2	6.2

[a] The calculation was performed considering the particle as a homogeneous matrix.
[b] From the quantitative analysis of the ESCA spectra.

3.5. Binding of plasmid DNA to SLNs

The agarose gel electrophoresis analysis was used to test the SLN-SA formulation for its ability to bind DNA via electrostatic interactions before and after the freeze-drying process. The aim of this investigation was to evaluate whether the decrease of the zeta potential, observed for the reconstituted freeze-dried powder, leads to the inability of the re-dispersed sample to bind gene material. Using the fresh prepared suspension (Fig. 7) the SLN:DNA were mixed in various ratios (w/w) i.e.: 20:1, 40:1, 60:1 and 80:1 corresponding to cationic lipid:DNA ratios (calculated from quantitative analysis of ESCA spectra) of 0.5:1, 0.9:1, 1.4:1 and 1.8:1, respectively. Regardless of the SLN:DNA ratios a strong DNA retention, indicating the formation of stable complexes, was observed (lanes 2–5). As transfection of cells in culture generally is performed in serum free medium (i.e. DMEM), it was important to assure that SLN:DNA complexes were stable in this environment. Thus, in order to check the effect of the

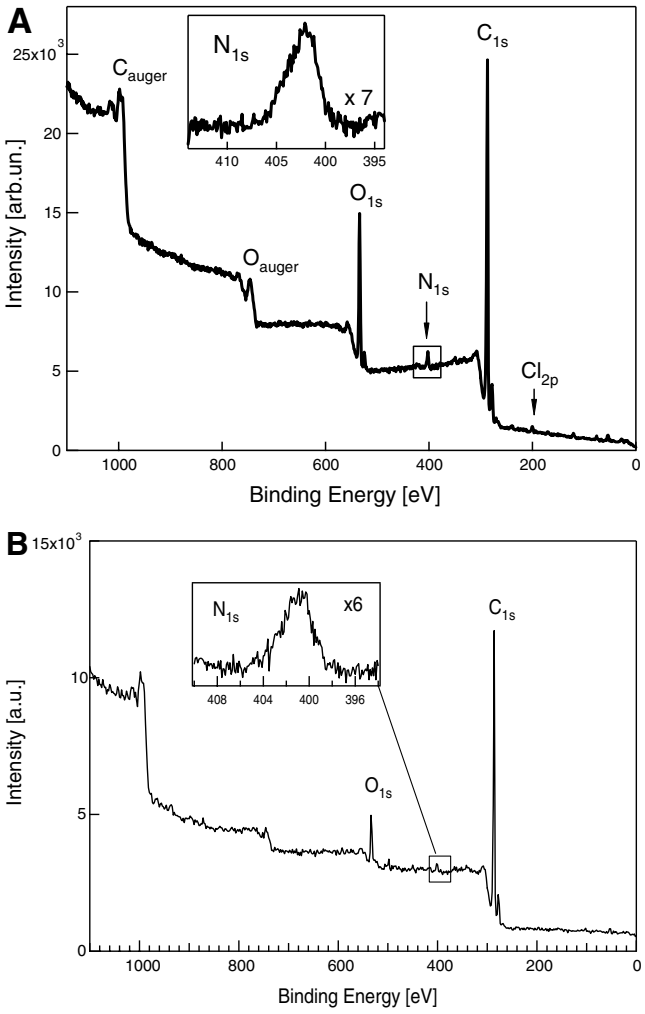

Fig. 6. ESCA spectrum of freshly prepared (spectrum A) and re-dispersed (spectrum B) cationic SLN-SA.

DMEM solution on the SLN:DNA complexes, the complexes formed according to the ratios indicated above were diluted in DMEM (lanes 6–9). The results obtained indicate that the preformed complexes were not perturbed by the addition of DMEM.

Regarding the re-dispersed freeze-dried SLN-SA, the following SLN:DNA ratios (w/w) were examined: 20:1, 40:1, 60:1, 80:1, corresponding to cationic lipid: DNA ratios of 0.4:1, 0.8:1, 1.2:1, and 1.6:1, respectively. In this case two kinds of re-suspended media were used: PBS (Fig. 8A) or deionised water (Fig. 8B). When the SLN-SA were re-suspended in PBS no formation of stable SLN:DNA complexes was observed, regardless of the ratios considered. These findings may be due to the presence of the phosphate anions that interact electrostatically with the positive charge of the particles preventing the interaction with the DNA. On the contrary, when freeze-dried SLN-SA were re-suspended in deionised water (Fig. 8B) the ability of the SLNs to complex DNA was restored in the same SLN:DNA ratios observed for the fresh prepared suspensions (lanes 2–5); moreover the

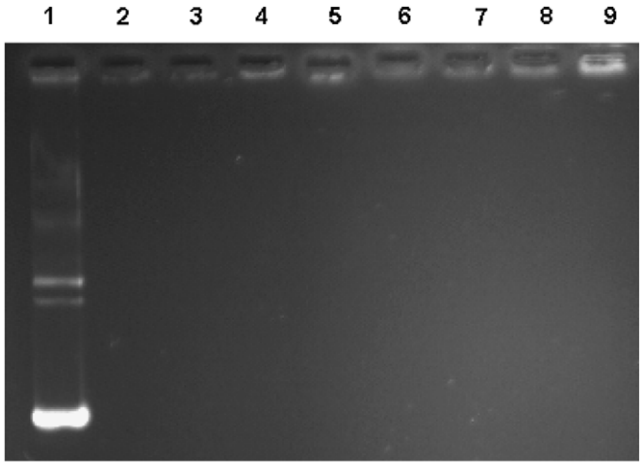

Fig. 7. Agarose gel electrophoresis analysis of SLN/pEGFP plasmid mixtures prepared using fresh prepared suspension of SLN-SA. Lanes from left: 1 naked plasmid DNA; 2–5 SLN/DNA mixtures diluted in water at different ratios: 20:1, 40:1, 60:1 and 80:1, respectively; 6–9 again SLN/DNA mixtures but diluted in DMEM without serum at 20:1, 40:1, 60:1 and 80:1 ratios, respectively.

formed complexes were not broken by the addition of DMEM (lanes 6–9).

Thus, the pEGFP plasmid was stably complexed by the SLN-SA either as fresh prepared suspension or as freeze-dried powder at the same SLN:DNA ratios. However, the SLN:DNA ratios from fresh prepared SLN-SA did not correspond to the same cationic lipid DNA ratios from the freeze-dried sample. This means that the loss of the cationic lipids amount observed for the freeze-dry process did not modify the ability of the carrier to bind DNA.

4. Conclusion

In the absence of cryoprotectors, the freeze-drying process did not modify the size and the morphology of the sample constituted of stearic acid (SLN-SA) whereas it leads to an irreversible particle aggregation for the particles based on Compritol or cetylpalmitate. Therefore, in the freeze-drying of SLNs, avoiding the use of lyo- or cryoprotectors can be realized only after an accurate selection of the materials. Moreover, even if the SLN-SA freeze-dried displayed a reduction of the positive surface charge, this does not influence the outcome of the DNA complexation that is an essential prerequisite for the DNA delivery.

Acknowledgements

Thanks are due to Fondazione Cassa di Risparmio di Modena for financial support. Thanks are also due to Dr. M. Tonelli for his technical support in the AFM analysis and to Dr. L. Rettighieri for his technical advice in the ESCA. The authors are also grateful to Prof. F. Forni for critical reading of the manuscript.

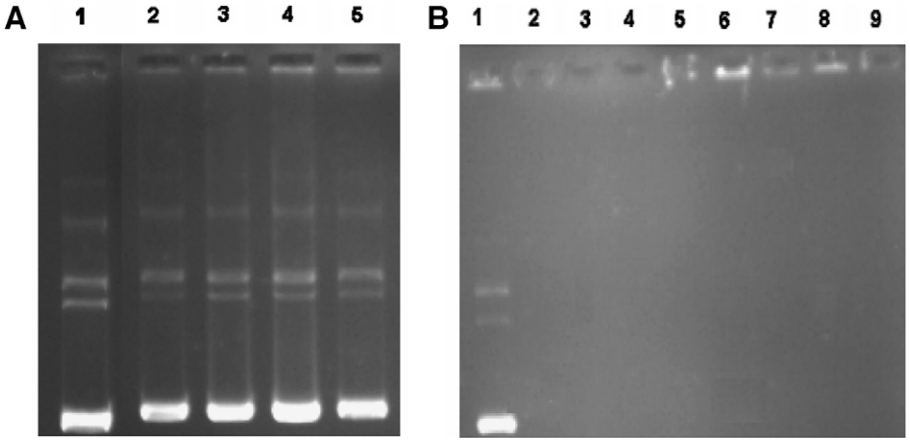

Fig. 8. A: Agarose gel electrophoresis of mixtures of pEGFP plasmid and freeze-dried SLN-SA reconstituted in Dulbecco's PBS. Lane from left: 1 naked DNA plasmid; 2–5 SLN/DNA mixtures with w/w ratio of 20:1, 40:1, 60:1 and 80:1, respectively. B: Agarose gel electrophoresis of mixtures of plasmid DNA and freeze-dried SLN-SA reconstituted in deionised water. Lane from left: 1 naked DNA plasmid; 2–5 SLN/DNA mixtures diluted in water with w/w ratio of 20:1, 40:1, 60:1 and 80:1, respectively; 6–9 SLN/DNA mixtures diluted in DMEM without serum with w/w ratio of 20:1, 40:1, 60:1 and 80:1, respectively.

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